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# Daytime Variation in Serum Progesterone During the Mid-Luteal Phase in Women Undergoing *In Vitro* Fertilization Treatment

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**Objective:** To investigate whether mid-luteal serum progesterone (P<sub>4</sub>) exhibits significant fluctuations during a 12-h daytime period in women undergoing *in vitro* fertilization (IVF) and to explore whether the extent of these fluctuations could impact the interpretation of luteal progesterone levels in a clinical setting.

**Design:** Explorative pilot study.

**Setting:** Public hospital-based fertility unit.

**Patients:** Ten women undergoing IVF treatment.

**Intervention:** Seven days after oocyte pick-up, patients underwent frequent repeated blood sampling (every 60 min for 12 h and during two of these hours, every 15 min). Serum samples were analyzed for progesterone, estradiol, and luteinizing hormone (LH).

**Main outcome measures:** Daytime fluctuations in s-progesterone and s-estradiol.

**Results:** There was a significant positive correlation between median P<sub>4</sub> levels and the magnitude of P<sub>4</sub> variations—women with median P<sub>4</sub> < 60 nmol/l had clinically stable P<sub>4</sub> levels throughout the day, while patients with median P<sub>4</sub> > 250 nmol/l exhibited periodic P<sub>4</sub> peaks of several hundred nanomoles per liter. These endogenous P<sub>4</sub> fluctuations were observed irrespective of the type of stimulation protocol or mode of triggering of final oocyte maturation and despite the fact that LH was under the detection limit at the time of measurement. Simultaneously, large fluctuations were seen in s-estradiol.

**Conclusion:** Monitoring of early to mid-luteal P<sub>4</sub> levels in IVF cycles may be valuable in the planning of individualized luteal phase support in the attempt to increase reproductive outcomes. The prerequisite for luteal phase monitoring is, however, that the validity of a single measured P<sub>4</sub> value is reliable. We show for the first time, that a single P<sub>4</sub> measurement in the low progesterone patient quite accurately reflects the corpus luteum function and that the measurement can be used to detect IVF patients with a need of additional exogenous luteal P<sub>4</sub> administration.

**Keywords:** serum progesterone, *in vitro* fertilization, serum estradiol, luteal phase, daytime variation

## INTRODUCTION

The human corpus luteum (CL) is a transient ovarian endocrine gland, which is active during the luteal phase of the menstrual cycle and in early pregnancy until gestational week 8. The CL produces significant amounts of progesterone ( $P_4$ ), estradiol ( $E_2$ ), and androgens as well as growth factors and nonsteroidal hormones. The overall maintenance of CL function is critically dependent upon regular stimulation of pituitary luteinizing hormone (LH) or human chorionic gonadotropin (hCG) to sustain the steroidogenesis from the luteinized granulosa and theca cells (1). A sufficient  $P_4$  production from the CL is an absolute necessity for the decidualization of the endometrium preceding implantation and the establishment of early pregnancy. Progesterone secretion from the CL is maximal during the mid-luteal phase inducing a serum  $P_4$  level of approximately 40–60 nmol/l in the natural cycle (2, 3).

During ovarian stimulation for *in vitro* fertilization (IVF) supra-physiological levels of  $E_2$  are obtained during the late follicular phase as a result of the multifollicular growth. This hyper-estrogenic state must be counterbalanced in the luteal phase by an increased  $P_4$  load to achieve a receptive endometrium in time for embryo transfer. Previously, Humaidan and co-workers showed that the use of GnRH agonist trigger in IVF cycles followed by a standard vaginal luteal phase support resulted in mid-luteal  $P_4$  levels comparable to levels seen in the natural cycle (39 nmol/l) (4). However, in contrast to what was expected, this  $P_4$  level was too low to secure successful implantation and pregnancy, resulting in an ongoing IVF pregnancy rate of only 6%. Thus, emphasizing the fact that the  $P_4$  requirement during the luteal phase of the stimulated cycle is greater than that of the natural cycle. When the luteal phase support was modified by adding a bolus of 1,500 IU hCG on the day of oocyte retrieval, the mid-luteal  $P_4$  level of the GnRHa triggered cycle increased to 74 nmol/l resulting in a delivery rate of 24% per transfer (5). It seems that a mid-luteal serum  $P_4$  threshold of approximately 80–100 nmol/l exists after IVF treatment followed by fresh embryo transfer, and that this threshold must be surpassed in order to secure a successful reproductive outcome (6). The traditional luteal phase support in artificial IVF cycles with administration of vaginal micronized  $P_4$  induces a luteal serum  $P_4$  level of approximately 40 nmol/l (7–9). Thus, a substantial additional endogenous  $P_4$  production by the CL is mandatory to surpass the  $P_4$  threshold to subsequently optimize the chance of pregnancy following IVF treatment. Traditionally, clinicians do not monitor the luteal phase  $P_4$  levels in the firm belief that the luteal phase support will cover the  $P_4$  need of the cycle. However, we have previously seen that more than 25% of IVF patients in both the hCG and GnRHa triggered group have a mid-luteal serum  $P_4$  below 60 nmol/l despite luteal phase support and the fact that they had more than 14 follicles on the day of aspiration (10). Furthermore, data from non-human species (11, 12) and data from human frozen/thawed embryo cycles (13, 14) have shown that an optimal luteal  $P_4$  range exists and that pregnancy outcome is reduced not only below but also above this optimal  $P_4$  level. Whether this is also the case following IVF and fresh embryo transfer, is still to be explored. If this is the case, monitoring of luteal  $P_4$  levels may help to improve the

reproductive outcome in IVF cycles by allowing an individualization of treatment based on the serum  $P_4$  measurements.

However, mid-luteal  $P_4$  measurements are complicated by the pulsatile nature of hormone secretion from the CL. Filicori and co-workers (15) showed that plasma  $P_4$  concentrations exhibit large and rapid fluctuations during the mid-luteal phase of naturally cycling women. Thus,  $P_4$  levels ranged from values as low as 7 nmol/l to peaks of 128 nmol/l within minutes during a 24-h study period. In the natural cycle, two distinguishable types of  $P_4$  pulses exist during the mid-luteal phase: those preceded by an LH pulse and others emerging at time of LH quiescence; the latter being a result of an autonomous steroid secretion by the CL independent of LH activity. During the mid-luteal phase of the stimulated IVF cycle, the pituitary is suppressed by the negative feedback from supra-physiological steroid levels and s-LH is significantly reduced to levels much lower (0.5–0.7 IU/l) than seen in the mid-luteal phase of the natural cycle (5–7 IU/l) (16–18). How this diminished LH pulse activity influences the secretory pattern of ovarian steroidogenesis during the mid-luteal phase of an IVF cycle is until now unknown.

The present study was performed to explore whether mid-luteal serum  $P_4$  levels in an IVF cycle exhibit a similar high-pulsatile pattern as seen during the natural cycle, knowing that the LH pulse activity is distinctly reduced. From a clinical point of view, we wanted to investigate whether a single morning  $P_4$  measurement provided a reliable index of mid-luteal CL function following IVF treatment.

## MATERIALS AND METHODS

### Study Population

Ten female patients undergoing IVF/ICSI at the Fertility Clinic in Skive, Denmark, from December 2014 to December 2015 volunteered to participate in the study. Clinical information regarding age, body mass index (BMI), smoking habits, biochemical reproductive profile, cause of infertility, prior IVF attempts, course of stimulation, and laboratory results were recorded. Baseline characteristics of participants are provided in **Table 1**. Written informed consent was obtained from all patients prior to study participation. Participants were chosen so as to represent both the long GnRH agonist cycle as well the GnRH antagonist cycle and different types of triggering for final oocyte maturation (hCG or GnRH agonist).

### Protocols for Ovarian Stimulation

Six patients were treated in a long GnRH agonist cycle with pituitary suppression using SC injection of Buserelin 0.8 mg (Suprefact®; Sanofi, Denmark) starting in the mid-luteal phase of the preceding cycle. A daily dose of 0.4 mg Buserelin was administered until the day before ovulation triggering. On day 2 of the cycle, a transvaginal ultrasound examination was carried out, and in case of an endometrial thickness < 4 mm, ovarian stimulation started with corifollitropin-alfa (Elonva®; MSD, Denmark) in combination with either r-FSH/rLH (Pergoveris®; Merck Biopharma, Denmark) or hMG (Menopur®, Ferring Pharmaceuticals, Denmark). The gonadotropin dosage was

**TABLE 1** | Description of demographic data, ovarian stimulation, luteal phase support, and progesterone levels in study patients.

Patient	Age (years)	Body mass index (kg/m <sup>2</sup> )	Basal FSH (IU/l)	Cause of infertility	Protocol	Total FSH sum (IU)	Duration of FSH stimulation (days)	Ovulation trigger	Luteal phase support	No. of follicles > 14 mm	No. of oocytes	No. of MII	Mid-luteal P <sub>4</sub> (nmol/l)
1	38	24.4	6.5	Male factor	Long GnRHa	3,225	11	hCG 10,000 IU	Lutinus 300 mg daily	5	5	1	89
2	37	22.8	6.6	No male partner/ endometriosis	GnRH antagonist	1,725	9	Suprefact 0.5 mg	Lutinus 300 mg daily + 1,500 hCG (OPU) + 1,000 hCG (OPU + 5)	10	11	10	283
3	39	30.0	6.4	No male partner	Long GnRHa	3,450	12	Ovitrelle 6,500 IU	Lutinus 300 mg daily	14	12	12	277
4	34	22.2	14.7	Unexplained	Long GnRHa	3,600	12	hCG 10,000 IU	Lutinus 300 mg daily	9	8	8	213
5	28	19.9	5.9	Male factor	Long GnRHa	2,925	11	hCG 10,000 IU	Lutinus 300 mg daily	9	6	3	97
6	37	25.3	8.3	Unexplained	Long GnRHa	3,600	12	Ovitrelle 6,500 IU	Lutinus 300 mg daily	11	8	8	376
7	28	31.8	3.9	Endometriosis	GnRH antagonist	2,025	9	Suprefact 0.5 mg	Crinone 180 daily + 1,500 hCG (OPU)	17	11	11	36
8	36	25.6	4.9	Tubal factor	GnRH antagonist	1,913	9	Suprefact 0.5 mg	Crinone 180 daily + 1,500 hCG (OPU)	19	13	13	55
9	40	34.5	7.2	Unexplained	Long GnRHa	2,250	9	hCG 10,000 IU	Lutinus 300 mg daily	7	6	6	54
10	36	29.4	7.2	Male factor	GnRH antagonist	3,938	15	Suprefact 0.5 mg	Lutinus 300 mg daily + 1,000 hCG (OPU) + 500 (OPU + 5)	20	14	8	161

Mid-luteal P<sub>4</sub> = median progesterone level (nmol/l) 7 days after oocyte retrieval.  
hCG, human chorionic gonadotropin; OPU, oocyte pick-up.

determined individually based on patient age, BMI, baseline FSH, previous response to gonadotropins, and antral follicle count and adjusted by monitoring follicular size by transvaginal ultrasound during treatment. Final oocyte maturation was induced with either hCG 10,000 IU SC (Pregnyl®, MSD, Denmark) or 6,500 IU SC (Ovitrelle®, Merck Biopharma, Denmark) when two or more leading follicles reached a mean diameter of 17 mm. Oocyte retrieval was carried out 36 h after hCG administration. IVF/ICSI procedures and embryo culture were performed according to normal clinical practice. A maximum of two embryos were transferred on day 3 or day 5 after oocyte retrieval. Luteal phase support was given as vaginal micronized P<sub>4</sub> (Lutinus® 300 mg daily, Ferring Pharmaceutical, Denmark or Crinone® 180 mg daily, Merck Biopharma, Denmark) starting 1 day after oocyte pick-up (OPU).

In four patients the GnRH antagonist protocol was used. On day 2 of the cycle ovarian stimulation commenced with either r-FSH (Gonal-F®, Merck Biopharma, Denmark) or hMG (Menopur®, Ferring Pharmaceuticals, Denmark) after a vaginal ultrasound examination. Daily GnRH antagonist co-treatment (Orgalutran® 0.25 mg/day, MSD, Denmark) was added at a follicle size of 12 mm. The FSH dose was individually adjusted according to the ovarian response. Final oocyte maturation was induced with SC Buserelin 0.5 mg (Suprefact®, Sanofi, Denmark) as soon as two or more follicles of  $\geq 17$  mm were present. Oocyte retrieval was carried out 36 h later. A maximum of two embryos were transferred on day 3 or day 5 after OPU. Luteal phase support was given in an individualized regimen consisting of vaginal administration of 300 mg micronized P<sub>4</sub> daily (Lutinus®, Ferring Pharmaceuticals, Denmark) in combination with a bolus of hCG (1,000–1,500 IU) on the day of oocyte retrieval (5, 10). Based on the individual ovarian response to stimulation, some patients received an additional hCG bolus on OPU + 5 (500–1,000 IU)

(10). See Table 1 for details. Vaginal P<sub>4</sub> administration continued until the day of pregnancy testing (hCG trigger) or until seventh gestational week (GnRHa trigger).

## Blood Sampling

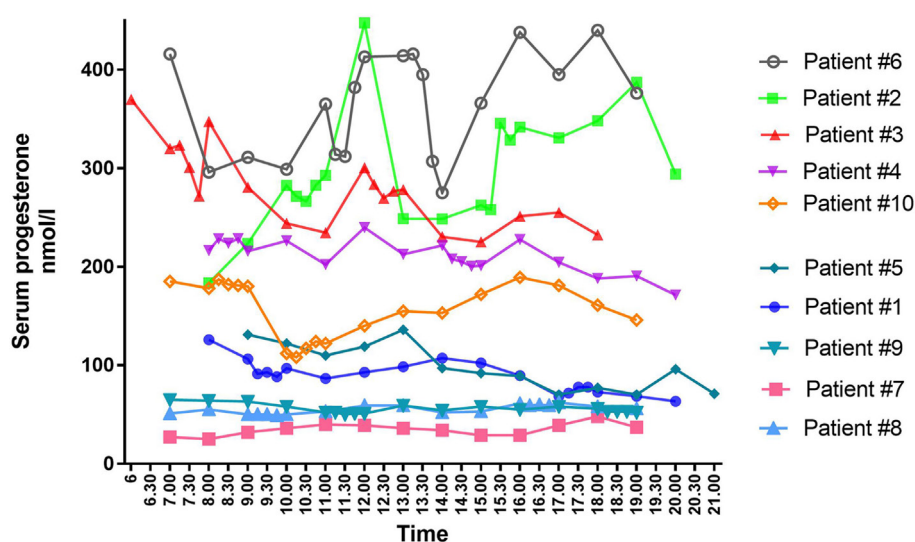
Blood sampling was conducted during the mid-luteal phase, i.e., 7 days after OPU (OPU + 7). Patients were admitted to the fertility unit early in the morning and stayed at the clinic for the subsequent 12 h. The starting time for blood sampling was between 6 a.m. and 9 a.m. for all patients. Participants were allowed normal daily life activities during the study period.

An intravenous cannula was inserted into a vein in the ante-cubital fossa and blood samples (4 ml) were drawn every 60 min for 12 h ( $n = 10$ ) and for two of these hours every 15 min ( $n = 8$  because of difficult venous access in two patients). After coagulation at room temperature, blood samples were centrifuged and serum was isolated and stored at  $-80^{\circ}\text{C}$  until analysis.

## Hormone Measurement

Serum P<sub>4</sub> (nmol/l), E<sub>2</sub> (pmol/l), and LH (IU/l) concentrations were measured using automated electro chemiluminescent immunoassays (Cobas® Modular analytics E170, Roche Diagnostics, Switzerland) routinely used for analysis at Department of Biochemistry, Viborg Regional Hospital, Denmark. All measurements were performed according to manufacturer's instructions using a commercially available chemiluminescent immunoassay kit intended for measurements in serum.

The detection limit of hormones was 0.2 nmol/l, 18.4 pmol/l, and 0.1 IU/l for P<sub>4</sub>, E<sub>2</sub>, and LH, respectively. All serum samples from each patient were measured within the same assay run. All hormone concentrations above the assay detection limit were measured in duplicate. The intra-assay coefficients of variation for P<sub>4</sub>, E<sub>2</sub>, and LH were all below 4%.



**FIGURE 1** | Individual mid-luteal serum profiles of progesterone over a 12-h interval in 10 patients undergoing controlled ovarian stimulation for *in vitro* fertilization treatment.



## Statistics

Data are presented as mean  $\pm$  SD or median and range when appropriate. The maximum absolute variation (MAV) in serum  $P_4$  over a 12-h period is given as the maximum  $P_4$  concentration – minimum  $P_4$  concentration during the time of sampling for each patient.

Spearman's correlation coefficient ( $r$ ) was calculated to correlate median steroid levels with the maximum absolute hormone variation during the day (MAV). A  $p$  value  $< 0.05$  was considered to be statistically significant. All analyses were performed using STATA, version 13.

## Ethics

The study was conducted according to the declaration of Helsinki for Medical Research and approved by the local Ethics Committee of the Central Denmark Region. ClinicalTrials.gov registration number NCT02673034.

## RESULTS

### Patient Characteristics

Patients had a mean age of  $35.3 \pm 4.2$  years, mean BMI of  $26.6 \pm 4.7$  kg/m<sup>2</sup> and  $1.9 \pm 2.0$  prior IVF attempts. Median level of FSH for all patients was 6.55 UI/l (interquartile range 5.9;7.2 IU/l). All participants were non-smokers. In four patients the cause of infertility was non-female (male factor or no male partner), in three patients the cause was female (tubal factor or endometriosis) and in three patients the cause of infertility was idiopathic (unexplained). See Table 1 for details.

### Overall Mid-Luteal Progesterone Values

Three patients had median mid-luteal  $P_4$  levels below 60 nmol/l. Two of these patients (#7 and #8) were triggered with a GnRH agonist and received luteal phase support with 1,500 IU hCG (OPU) and vaginal  $P_4$  (Crinone 180 mg daily). Despite having 19 and 17 follicles  $\geq 14$  mm and 11–13 mature oocytes retrieved at the day of OPU they presented with a mid-luteal  $P_4$  of only 55 and 36 nmol/l, respectively. The other patient (#9) with  $P_4 < 60$  nmol/l was triggered with 10,000 IU hCG and had seven follicles  $> 14$  mm at the day of aspiration.

### Overall Mid-Luteal LH Values

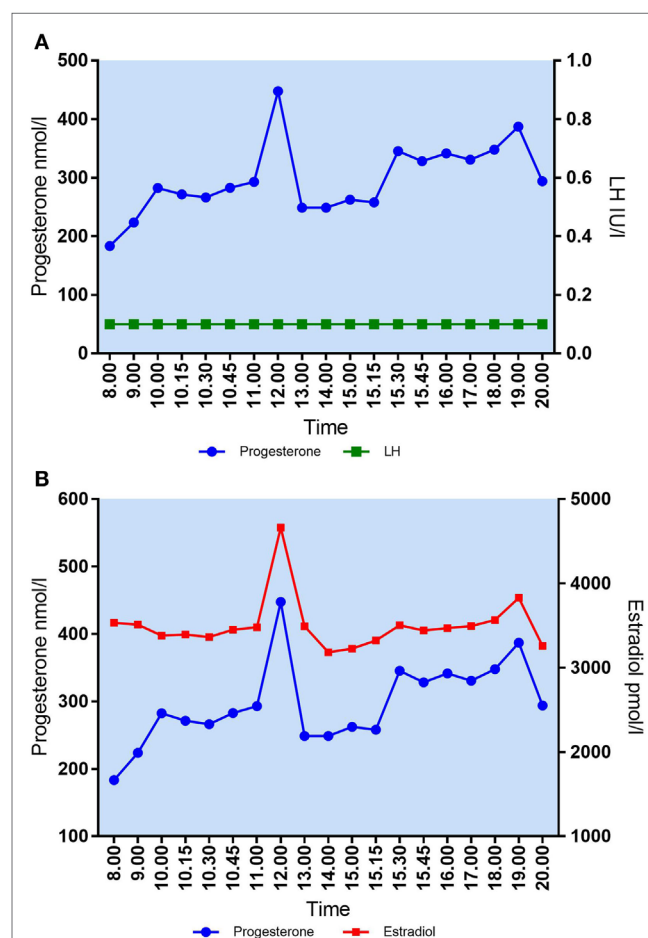
None of the patients downregulated in a long GnRH agonist protocol ( $n = 6$ ) had s-LH levels above the detection limit of the assay at any point during measurement (i.e., LH  $< 0.1$  IU/l). In three of the four patients stimulated in the GnRH antagonist protocol a modest LH pulse activity was seen with LH amplitudes ranging from 0.2 to 2.8 UI/l. In all patients, the LH peak was followed by an increase in serum  $P_4$  ranging from 4 to 36 nmol/l.

### Daytime Variation in Serum Progesterone

As seen during the natural cycle, large fluctuations in mid-luteal  $P_4$  were also present during daytime in some of the women undergoing IVF treatment (Figure 1). Fluctuations in luteal steroids were seen independent of the choice of stimulation protocol, the mode of final oocyte maturation and the type of luteal phase support.

The largest variation in  $P_4$  levels was seen in patients with median  $P_4 > 250$  nmol/l. In patient #2 with a median  $P_4$  of 283 nmol/l,  $P_4$  fluctuated from 293 nmol/l at 11 a.m. to 448 nmol/l at 12 p.m.—i.e., an increase of 155 nmol/l within 1 h. This fluctuation in  $P_4$  level was present even though s-LH was under the detection level throughout the day (Figure 2A). The increase in  $P_4$  was accompanied by a comparable increase in  $E_2$  (Figure 2A). Serum  $P_4$  concentrations during the 12-h period for that specific patient ranged from 183 nmol/l early in the morning to 448 nmol/l during the day—thus, a MAV during the study period of  $\Delta 265$  nmol/l. In patient #6 (median  $P_4$  376 nmol/l) and #3 (median  $P_4$  277 nmol/l) a rapid elevation of  $P_4$  levels ( $\Delta 70$ –75 nmol/l, respectively) was seen within a period of only 15 min without any concomitant LH activity (LH  $< 0.1$  IU/l). In comparison, patient #7 had a median  $P_4$  of only 36 nmol/l and showed only minor fluctuations throughout the day with  $P_4$  levels ranging from 25 to 48 nmol/l following a small detectable increase in LH secretion (see Table 2 for complete daytime  $P_4$  values).

There was a positive correlation between median  $P_4$  levels and MAV in  $P_4$  during daytime (Spearman's  $r = 0.9273$ ,  $p = 0.0001$ ).



**FIGURE 2 | (A)** Daytime variation in mid-luteal s-progesterone and s-LH in patient #2. Median  $P_4 = 283$  nmol/l. Maximal variation  $P_4/12$  h = 265 nmol/l. **(B)** Daytime variation in mid-luteal s-progesterone and s-estradiol in patient #2. Median  $E_2 = 3,471$  pmol/l. Maximal variation  $E_2/12$  h = 1,481 pmol/l.

**TABLE 2** | Mid-luteal serum progesterone concentrations during daytime in 10 women undergoing *in vitro* fertilization treatment.

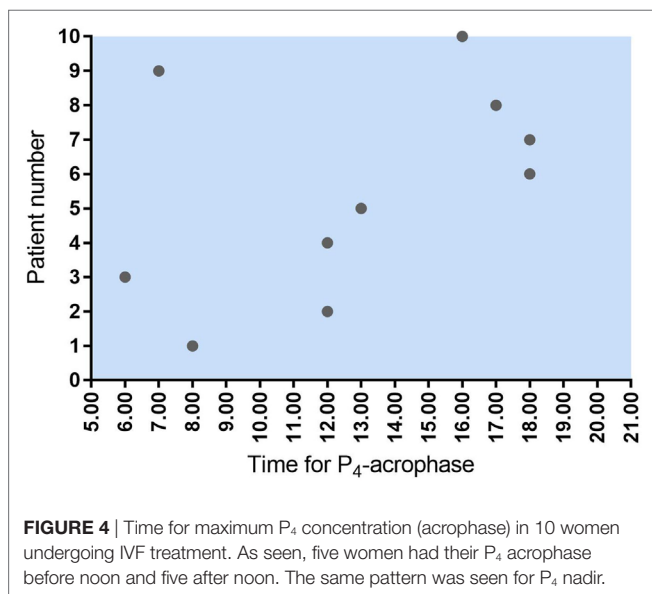
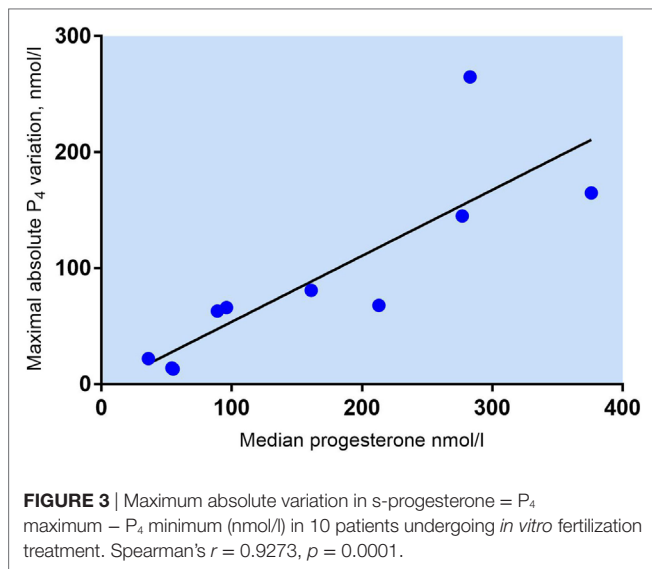
Patient											Median P <sub>4</sub> (range)
<b>1</b>	Time	8.00 a.m.	9.00 a.m.	9.15 a.m.	9.30 a.m.	9.45 a.m.	10.00 a.m.	11.00 a.m.	12.00 p.m.	13.00 p.m.	14.00 p.m.
	P <sub>4</sub> nmol/l	126	106	91	93	88	97	87	93	99	107
		15 p.m.	16 p.m.	17 p.m.	17.15 p.m.	17.30 p.m.	17.45 p.m.	18 p.m.	19 p.m.	20 p.m.	
		102	89	69	72	78	78	73	67	63	
											89 (63–126) ΔP <sub>4</sub> : 63
<b>2</b>	Time	8.00 a.m.	9.00 a.m.	10.00 a.m.	10.15 a.m.	10.30 a.m.	10.45 a.m.	11.00 a.m.	12.00 p.m.	13.00 p.m.	14.00 p.m.
	P <sub>4</sub> nmol/l	183	224	283	271	266	283	293	448	249	249
		15.00 p.m.	15.15 p.m.	15.30 p.m.	15.45 p.m.	16.00 p.m.	17.00 p.m.	18.00 p.m.	19.00 p.m.	20.00 p.m.	
		262	258	345	328	342	331	348	387	294	
											283 (183–448) ΔP <sub>4</sub> : 265
<b>3</b>	Time	6.00 a.m.	7.00 a.m.	7.15 a.m.	7.30 a.m.	7.45 a.m.	8.00 a.m.	9.00 a.m.	10.00 a.m.	11.00 a.m.	12.00 p.m.
	P <sub>4</sub> nmol/l	370	320	323	301	272	347	281	244	235	301
		12.15 p.m.	12.30 p.m.	12.45 p.m.	13.00 p.m.	14.00 p.m.	15.00 p.m.	16.00 p.m.	17.00 p.m.	18.00 p.m.	
		283	269	277	278	230	225	251	255	232	
											277 (225–370) ΔP <sub>4</sub> : 145
<b>4</b>	Time	8.00 a.m.	8.15 a.m.	8.30 a.m.	8.45 a.m.	9.00 a.m.	10.00 a.m.	11.00 a.m.	12.00 p.m.	13.00 p.m.	14.00 p.m.
	P <sub>4</sub> nmol/l	216	228	224	228	216	226	202	240	213	222
		14.15 p.m.	14.30 p.m.	14.45 p.m.	15.00 p.m.	16.00 p.m.	17.00 p.m.	18.00 p.m.	19.00 p.m.	20.00 p.m.	
		208	205	200	201	228	204	188	190	171	
											213 (171–240) ΔP <sub>4</sub> : 69
<b>5</b>	Time	9.00 a.m.	10.00 a.m.	11.00 a.m.	12.00 p.m.	13.00 p.m.	14.00 p.m.	15.00 p.m.	16.00 p.m.	17.00 p.m.	18.00 p.m.
	P <sub>4</sub> nmol/l	131	122	110	119	136	97	92	89	70	77
		19.00 p.m.	20.00 p.m.	21.00 p.m.							
		70	96	71							
											97 (70–136) ΔP <sub>4</sub> : 66
<b>6</b>	Time	7.00 a.m.	8.00 a.m.	9.00 a.m.	10.00 a.m.	11.00 a.m.	11.15 a.m.	11.30 a.m.	11.45 a.m.	12.00 p.m.	13.00 p.m.
	P <sub>4</sub> nmol/l	416	296	311	299	365	314	312	382	413	414
		13.15 p.m.	13.30 p.m.	13.45 p.m.	14.00 p.m.	15.00 p.m.	16.00 p.m.	17.00 p.m.	18.00 p.m.	19.00 p.m.	
		416	395	307	275	366	438	395	440	376	
											376 (275–440) ΔP <sub>4</sub> : 165
<b>7</b>	Time	7.00 a.m.	8.00 a.m.	9.00 a.m.	10.00 a.m.	11.00 a.m.	12.00 p.m.	13.00 p.m.	14.00 p.m.	15.00 p.m.	16.00 p.m.
	P <sub>4</sub> nmol/l	27	25	32	36	40	39	36	34	29	29
		17.00 p.m.	18.00 p.m.	19.00 p.m.							
		39	48	37							
											36 (25–48) ΔP <sub>4</sub> : 23
<b>8</b>	Time	7.00 a.m.	8.00 a.m.	9.00 a.m.	9.15 a.m.	9.30 a.m.	9.45 a.m.	10.00 a.m.	11.00 a.m.	12.00 p.m.	13.00 p.m.
	P <sub>4</sub> nmol/l	51	55	50	50	50	49	50	53	59	59
		14.00 p.m.	15.00 p.m.	16.00 p.m.	16.15 p.m.	16.30 p.m.	16.45 p.m.	17.00 p.m.	18.00 p.m.	19.00 p.m.	
		52	53	61	59	60	59	62	58	56	
											55 (49–62) ΔP <sub>4</sub> : 13
<b>9</b>	Time	7.00 a.m.	8.00 a.m.	9.00 a.m.	10.00 a.m.	11.00 a.m.	11.15 a.m.	11.30 a.m.	11.45 a.m.	12.00 p.m.	13.00 p.m.
	P <sub>4</sub> nmol/l	65	64	63	58	52	52	50	51	51	59
		14.00 p.m.	15.00 p.m.	16.00 p.m.	17.00 p.m.	18.00 p.m.	18.15 p.m.	18.30 p.m.	18.45 p.m.	19.00 p.m.	
		54	58	55	58	56	53	53	53	52	
											54 (51–65) ΔP <sub>4</sub> : 14
<b>10</b>	Time	7.00 a.m.	8.00 a.m.	8.15 a.m.	8.30 a.m.	8.45 a.m.	9.00 a.m.	10.00 a.m.	10.15 a.m.	10.30 a.m.	10.45 a.m.
	P <sub>4</sub> nmol/l	185	178	187	182	181	180	112	108	117	124
		11.00 a.m.	12.00 p.m.	13.00 p.m.	14.00 p.m.	15.00 p.m.	16.00 p.m.	17.00 p.m.	18.00 p.m.	19.00 p.m.	
		122	140	155	153	172	189	18	161	146	
											161 (108–189) ΔP <sub>4</sub> : 81

ΔP<sub>4</sub> = individual maximum absolute variation in P<sub>4</sub> during daytime = maximum P<sub>4</sub> – minimum P<sub>4</sub> concentration (nmol/l).

P<sub>4</sub> SI conversion factor: nmol/l = 3.18\*ng/ml.

The magnitude of P<sub>4</sub> pulses and thus the maximum variation is dependent on the median mid-luteal P<sub>4</sub> concentration (**Figure 3**). In patients with median P<sub>4</sub> > 250 nmol/l, very large fluctuations in serum P<sub>4</sub> were seen during daytime with a median MAV

of 165 nmol/l (range 145–265 nmol/l). Patients with median P<sub>4</sub> between 89 and 213 nmol/l had median MAV of 68 nmol/l (range 63–81 nmol/l), whereas patients with very low mid-luteal P<sub>4</sub> levels (median P<sub>4</sub> < 60 nmol/l) had fairly constant serum



$P_4$  levels throughout the day (median MAV 14 nmol/l, range 13–23 nmol/l).

There was no common general daytime rhythm for  $P_4$  in the 10 women examined, suggesting that the luteal phase is patient specific. Some patients had their highest hormone levels in the morning—others peaked during the day or in the early evening (see **Figure 1**). The time of  $P_4$  acrophase (zenith) and  $P_4$  nadir was before noon in half of the patients and after noon in the other half of patients (**Figure 4**).

### Daytime Variation in Serum Estradiol

Large fluctuations in mid-luteal serum  $E_2$  were also seen during the 12-h sampling time. In patient #2,  $E_2$  increased from 3,480 to 4,664 pmol/l in 1 h ( $\Delta 1,184$  nmol/l) (**Figure 2B**). Patients had

individual maximum  $E_2$  variations (Max  $E_2$  – Min  $E_2$ ) over 12 h ranging from  $\Delta 404$  to  $\Delta 1,481$  pmol/l. There was no correlation between median  $E_2$  levels and MAV in mid-luteal  $E_2$  (Spearman's  $r = 0.4424$ ,  $p = 0.20$ ).

As expected,  $P_4$  and  $E_2$  seem to be co-secreted from the CL showing similar patterns of fluctuations over time (**Figure 5**). Patients with median  $P_4 < 60$  nmol/l had  $E_2$  ranging from 541 to 1,552 pmol/l (median  $E_2$  1,457 pmol/l) whereas patients with median  $P_4$  between 89 and 213 nmol/l had  $E_2$  levels from 659 to 4,884 pmol/l (median  $E_2$  2,843 pmol/l). In patients with median  $P_4 > 250$  nmol/l,  $E_2$  ranged from 3,471 to 3,919 pmol/l (median  $E_2$  3,874 pmol/l). There was a significant correlation between median  $P_4$  levels and median  $E_2$  levels during mid-luteal phase of the stimulated cycle (Spearman's  $r = 0.8424$ ,  $p = 0.002$ ).

## DISCUSSION

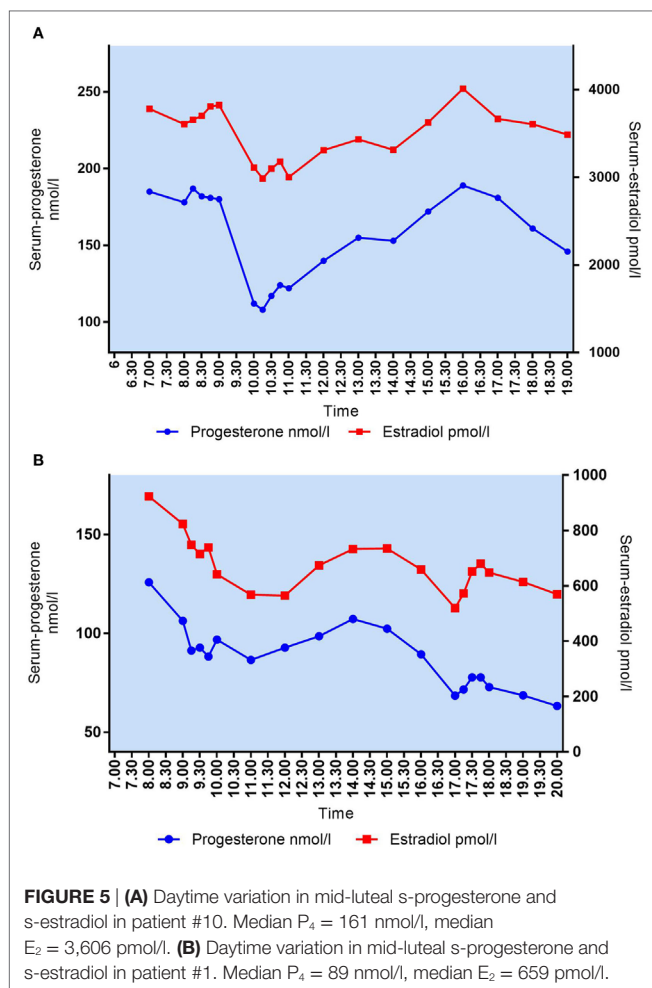
To the best of our knowledge, this is the first study to explore a possible daytime variation in  $P_4$  secretion during the mid-luteal phase in a group of women undergoing IVF treatment.

We found that the magnitude of mid-luteal  $P_4$  fluctuations following IVF treatment was dependent on the median  $P_4$  level. The largest  $P_4$  variations were seen in patients with median  $P_4$  exceeding 250 nmol/l (median MAV 165 nmol/l), whereas patients in the low  $P_4$  group (median  $P_4 < 60$  nmol/l) had relatively constant  $P_4$  levels throughout the day (median MAV 14 nmol/l). Patients showed a highly individual hormone secretion pattern without any obvious common daytime rhythm in  $P_4$  secretion. Serum  $E_2$  showed similar fluctuations in the mid-luteal phase with patients having individual  $E_2$  variations ranging from  $\Delta 404$  to  $\Delta 1,481$  pmol/l during the 12-h study time.

Earlier studies described the highly variable pattern of  $P_4$  secretion during the mid-luteal phase of naturally cycling women (15, 19, 20). These studies reported the presence of two distinguishable types of luteal  $P_4$  pulses—some preceded by a LH pulse and others non-concomitant to LH seen during time of pituitary quiescence. The latter seems to be the result of an autonomous  $P_4$  secretion from the CL, triggered and maintained by intraovarian concentrations of  $E_2$ , oxytocin, and PGF<sub>2 $\alpha$</sub>  (21, 22). The CL consists of two types of steroidogenic cells, i.e., the small luteal cells (SLCs) derived from follicular theca cells and the large luteal cells (LLCs) originating from follicular granulosa cells. Both the small and the large cells have extensive capacity to produce  $P_4$ . Moreover, both cells have unique steroidogenic functions and the “two-cell” mechanism of  $E_2$  biosynthesis appear to operate in the human CL analogous to the preovulatory follicle (23). Thus, the LLCs contain P450-aromatase essential for  $E_2$  synthesis whereas SLCs express P450c17 for androgen production (24, 25). Both types of luteal cells express  $E_2$  receptors (26) and  $E_2$  stimulation is a powerful trigger of  $P_4$  release from either cell type (27).

Isolation of large and SLCs from human corpora lutea has shown that once induced by the LH peak, the LLCs exhibit the greatest basal  $P_4$  production (28) and that this production is not increased by further LH stimulation (29). The LLCs produce  $P_4$  at a constant rate and are the dominant source of  $P_4$  during the





early luteal phase (30). During this period, the  $P_4$  levels exhibit a non-pulsatile pattern both in the natural (15) and stimulated cycle (OPU + 2) (31). The responsivity of the SLCs to LH/hCG develops during the early mid-luteal phase where cells respond with a pronounced increase in  $P_4$  secretion in response to LH pulses. The luteal  $P_4$  contribution from the SLCs increase during the mid- and late luteal phase during which  $P_4$  secretion becomes highly pulsatile (32).

Thus, the endogenous mid-luteal  $P_4$  level consist of three parts—the basal  $P_4$  production from the LLCs, the  $P_4$  pulses from SLCs triggered by pituitary LH, and the autonomous  $P_4$  fluctuations independent of luteotrophic stimuli.

Previously, Wuttke et al. proposed a model to explain the LH-independent fluctuations in mid-luteal  $P_4$  levels based on autocrine and paracrine mechanisms in the luteal tissue (21). Upon stimulation with LH during the mid-luteal phase, the SLCs start secretion of  $P_4$  as well as androstenedione—the latter is subsequently converted to  $E_2$  in LLCs by P450-aromatase. The increased  $E_2$  concentration acts in an autocrine way in LLCs to increase the release of both  $P_4$  and oxytocin. Oxytocin stimulates fibroblasts to release  $PGF_{2\alpha}$ , which in turn stimulates further oxytocin as well as  $E_2$  secretion from the luteal cells. The isolated effect of oxytocin and  $PGF_{2\alpha}$  upon the luteal cell lines is a decreased  $P_4$

secretion, but this effect is overridden by the concomitantly triggered increase in  $E_2$  which will elicit a pronounced  $P_4$  release. In this way, the LH pulse will stimulate an intra-luteal circuit involving auto- and paracrine effects of  $E_2$ , oxytocin,  $PGF_{2\alpha}$ —and possible a variety of other regulatory peptides, i.e., Substance P—and the net effect is the generation of a  $P_4$  pulse. This circuit functions for hours without further gonadotropic support, thus generating several  $P_4$  pulses with gradually decreasing amplitude until the next LH pulse sets off the intra-luteal  $E_2/P_4$  loop again. In contrast, in women with hypothalamic deficiency with suppressed LH levels and no LH pulses, mid-luteal  $P_4$  shows a non-pulsatile pattern, underlining the need for an initial high LH/hCG load to trigger the  $P_4$  circuit (21). The oxytocin induced  $P_4$  release can be prevented by treatment of the CL with tamoxifen—an estrogen receptor blocking agent—underlining the  $E_2$  regulation of the autonomous  $P_4$  pulses (27). This independent intra-luteal  $P_4$  pulse generator might serve as an additional biological safety mechanism preventing declining  $P_4$  levels in between LH pulses and might explain the function of the substantial  $E_2$  production during the luteal phase in humans.

In the stimulated IVF cycle, LH pulses are absent during the mid-luteal phase and serum LH levels are distinctly suppressed (33). The hCG bolus administered for ovulation induction or as luteal phase support exerts a tonic and constant stimulation on the luteal tissue due to the prolonged half-life of hCG and, therefore, cannot account for the rapid  $P_4$  fluctuations seen during the mid-luteal phase in this study. The standard vaginal  $P_4$  supplementation reaches steady state during the early luteal phase and contributes with remarkably constant serum  $P_4$  levels though out the day despite multiple daily vaginal doses (34). The very large fluctuations in serum  $P_4$  seen in the present study are, therefore, likely to be the result of the autonomous intraovarian  $P_4$  circuit. This is further emphasized by the fact that  $P_4$  peaks are accompanied by concomitant  $E_2$  rises and exogenous  $E_2$  was not provided as part of the luteal phase support.

We were not able to detect a common general pattern of  $P_4$  secretion during daytime in the 10 patients examined. The peak and nadir  $P_4$  levels occurred at different times in different patients, and the course of hormone levels during the day showed highly individual rhythms. This is in agreement with studies performed during the mid-luteal phase of the natural cycle (3, 19). In a study of seven women studied over 24 h in the mid-luteal phase of the natural cycle, the  $P_4$  acrophase varied from 10.31 a.m. to 11.33 p.m. (16). Based on the lack of a diurnal reproducible pattern for mid-luteal  $P_4$  in the IVF cycle the accuracy of the  $P_4$  measurement is not improved by a fixed timing of blood sampling and, thus, the  $P_4$  measurement could be performed at any time during clinic opening hours.

During the natural cycle both late follicular  $E_2$  levels, follicular diameter at the time of ovulation as well as area under the LH surge curve correlate poorly to the subsequent luteal phase  $P_4$  level (3). Thus, predicting patients with insufficient luteal  $P_4$  levels is troublesome based on the follicular development as abnormal luteal phases can be seen in cycles characterized by normal folliculogenesis (35). In the present study, the two patients with the lowest  $P_4$  levels (36 and 55 nmol/l) had 17 and 19 follicles, respectively, on the day of OPU, showing that a large number of CLs do not warrant

a high  $P_4$  output in the luteal phase. For this reason monitoring of luteal phase  $P_4$  could be of value to detect patients with low  $P_4$  levels, who might benefit from additional exogenous  $P_4$  therapy. However, the prerequisite for easy luteal phase monitoring is that the validity of a single measured  $P_4$  value is reliable and gives a reasonable estimate of the CL capacity of the patient.

We acknowledge that the small sample size of this study may limit the validity of general interpretations. However, we consider this explorative preliminary study to be pioneering as part of basic research and, importantly, it is the first to explore the mid-luteal  $P_4$  fluctuations in different types of IVF cycles. The autonomous LH-independent  $P_4$  bursts from the ovaries during the mid-luteal phase were seen in both GnRH analog types (GnRH antagonist and long GnRHa protocol) as well as after different types of triggering of final oocyte maturation (hCG or GnRH agonist). Thus, it seems that these autonomous episodic luteal  $P_4$  peaks are generated independently of the choice of treatment regimen and may, therefore, also apply to other IVF stimulation protocols.

## CONCLUSION

Based on the 10 women examined in this study, we state that the accuracy of a single mid-luteal serum progesterone measurement as an approximation of mean  $P_4$  levels throughout the day depends on the  $P_4$  concentration and that women with low  $P_4$  levels ( $P_4 < 60$  nmol/l) exhibit clinically stable  $P_4$  levels during daytime. Thus, a single  $P_4$  measurement in the low progesterone patient reflects quite accurately the CL function and a measured low  $P_4$  value can, therefore, be regarded as a “true low value.” Future studies should clarify, whether additional exogenous  $P_4$  support administered to the low luteal  $P_4$  patient group can improve the reproductive outcome.

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## DATA AVAILABILITY

The raw data supporting the conclusion of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## ETHICS STATEMENT

The study was conducted according to the declaration of Helsinki for Medical Research and approved by the local Ethics Committee of the Central Denmark Region. All patients provided written informed consent to participate in the study.

## AUTHOR CONTRIBUTIONS

LT designed and conducted the study. LT drafted the manuscript and UK, CA, and PH all contributed to the interpretation of data and critically reviewed the manuscript. All coauthors accepted the final draft.

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